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Assessment of Genetic Markers for Species Differentiation within the *Mycobacterium tuberculosis* Complex

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It is important to correctly identify species within the *Mycobacterium tuberculosis* complex because of the zoonotic implications of bovine tuberculosis, especially in developing countries. We assessed the use of various genetic markers for species-specific identification of mycobacteria from the *M. tuberculosis* complex. A multiplex PCR designed for detection of the *mtp40* and IS*1081* elements was optimized and evaluated in 339 mycobacterial strains from different animal and geographic origins. The host range of the IS*6110*, MPB70, and 16S rRNA genes was also studied by PCR in all the strains. Finally, the usefulness of the genetic markers was compared by an immunoperoxidase test for specific identification of *Mycobacterium bovis* strains. The *mtp40* sequence was detected in 87 of the 91 strains of *M. tuberculosis* and in 9 of the 11 *Mycobacterium africanum* strains but not in any of the *M. bovis* or *Mycobacterium microti* strains, indicating that the *mtp40*-IS*1081* multiplex PCR may be a useful test that could differentiate the accepted human pathogens *M. tuberculosis* and *M. africanum* from the known animal pathogens *M. bovis* and *M. microti*. Interestingly, the *mtp40* element was also found in all of the *M. tuberculosis* complex strains isolated from seals. This organism is considered to be a true seal pathogen, but its origin is essentially unknown. The finding of the *mtp40* element in the strains from seals suggests a closer relationship of these strains with a human origin than to an animal origin. The *mtp40* element was not found in any other mycobacterial species included in the study. As a result of this study, we suggest that biochemical tests or alternate genetic markers are still needed to differentiate *M. tuberculosis* from *M. africanum* when these species coexist as causative agents of tuberculosis. The immunoperoxidase test worked well for the identification of *M. bovis* strains. We also report, for the first time, PCR amplification of the repetitive element IS*6110* in an isolate of *Mycobacterium ulcerans* and an isolate of *Mycobacterium gilvum*, which emphasizes the need for further investigation of the host range of this sequence.

The *Mycobacterium tuberculosis* complex comprises the *Mycobacterium* species *M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum*, and *M. microti* and more recently the mycobacterial agent causing tuberculosis in seals (10). *M. tuberculosis* causes tuberculosis in humans, but infection has also been recorded in pet dogs and cats (1, 37) and other animals in contact with infected humans. *M. africanum* (6) causes tuberculosis in humans and primates (40). *M. microti*, the vole or dassie bacillus, causes tuberculosis in small rodents (11, 36, 43), and although it has been reported to cause infection in a cat and pigs (24) and in a llama (31), it is not considered to be an important human pathogen. *M. bovis* is the causative agent of bovine tuberculosis but has the widest host range of any of the members of the *M. tuberculosis* complex.

Because of the slow growth of *M. tuberculosis* complex organisms, identification by traditional methods (21) can take several weeks. Although some phenotypic characteristics can be used to separate them, different taxonomic studies (19, 25, 34) support the view that members of the *M. tuberculosis* complex should be recognized as a single species. However, the epidemiological differences between them make it convenient to classify them as separate species.

The incidence of human tuberculosis caused by *M. bovis* has decreased in developed countries following the pasteurization of milk for human consumption and the successful progress of bovine tuberculosis eradication campaigns. However, humans can act as reservoirs of *M. bovis*, and transmission of infection to cattle has been reported in several countries (2, 44).

Accurate information on the incidence of *M. bovis* infection in humans is limited in many countries. Studies in Argentina (3) and the southeast of England (45, 48) have reported incidences of between 0.4 and 6% of the total cases of human tuberculosis. According to data from the Pan American Health Organization and World Health Organization, 7,000 new cases of human tuberculosis caused by *M. bovis* arise in South America each year (29), and some authors have suggested that the real incidence could be eight times higher (33). In areas such as Africa, the zoonotic importance of *M. bovis* infection may be further exacerbated by closer contact with animals, consumption of nonpasteurized milk, and the increasing incidence of human immunodeficiency virus infections (15). *M. bovis* infection has been reported in several patients with AIDS in San Diego, Calif. (16), and a multidrug-resistant strain of *M. bovis* has been reported to have spread between patients in an AIDS ward in France (5).

M. tuberculosis, *M. bovis*, and *M. africanum* are able to cause tuberculosis in humans as well as in animals. The similarity in the clinical presentation and treatment of these infections has meant that many laboratories do not fully identify them (22). However, it is important to correctly identify these isolates so that epidemiological investigations can detect potential reservoirs that may be a risk to public health. Additionally, *M. bovis*

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TABLE 1. Mycobacterial isolates used in this study

Mycobacterium species	Source	No. of strains	Isolate details (*n*)
M. tuberculosis	Reference	1	NCTC 7416
M. tuberculosis	Humans	89	Australia (88), Spain (1)
M. tuberculosis	Dog	1	Spain
M. bovis	Reference	1	AN5
M. bovis	Cattle	99	Australia (59), Spain (30), Canada (9), Iran (1)
M. bovis	Goats	15	Spain
M. bovis	Deer, cat	3	Spain
M. bovis	Humans	43	Australia
M. bovis BCG	Reference	1	TMC 1029
M. bovis BCG	Reference, humans	16	United States (13), Australia (3)
M. africanum	Reference	4	TMC 3, 12, 37, and 54
M. africanum	Humans	7	Australia
M. microti	Reference, voles	3	NCTC 08710, United Kingdom (2)
M. microti	Rock hyrax	3	United Kingdom (1), Australia (2)
M. microti	Surikat	1	Sweden
M. tuberculosis complex	Seals	16	Australia
M. tuberculosis complex	Seal trainer	1	Australia
M. aurum	Reference	1	ATCC 23366
M. avium	Cattle	3	Australia (2), Spain (1)
M. avium	Human	1	Spain
M. chelonae	Reference	1	ATCC 19977
M. chitae	Reference	1	ATCC 19627
M. duvalii	Reference	1	NCTC 358
M. flavescens	Reference	1	ATCC 14474
M. fortuitum	Reference	1	NCTC 3631
M. gadium	Reference	1	ATCC 27726
M. gastri	Reference	1	ATCC 15754
M. genavense	Canary, parrot	2	Spain
M. gilvum	Reference	1	NCTC 10742
M. gordonae	Reference	1	ATCC 14470
M. intracellulare	Reference	1	ATCC 13950
M. kansasii	Reference	1	ATCC 12478
M. marinum	Reference	1	ATCC 927
M. neoaurum	Reference	1	ATCC 25795
M. nonchromogenicum	Reference	1	ATCC 19503
M. parafortuitum	Reference	1	ATCC 19686
M. phlei	Reference	1	ATCC 11758
M. scrofulaceum	Reference	1	ATCC 19981
M. terrae	Reference	1	ATCC 15755
M. thermoresistibile	Reference	1	ATCC 19527
M. triviale	Reference	1	ATCC 23292
M. ulcerans	Reference	1	ATCC 19423
M. vaccae	Reference	1	ATCC 15483
M. xenopi	Reference	2	NCTC 10042, ATCC 19276
M. xenopi	Humans	4	Australia

isolates are resistant to pyrazinamide, a drug commonly used for the treatment of tuberculosis (26).

Many laboratories now use molecular techniques for the identification of *M. tuberculosis* complex isolates. Acridinium ester-labelled DNA probes (AccuProbe; Gene Probe, Inc., San Diego, Calif.) are now available for this purpose (20, 28); however, these probes cannot distinguish between members of the complex.

PCR has also been used for the rapid identification of mycobacteria. Primers directed at the 16S rRNA gene can be used to identify organisms belonging to the genus *Mycobacterium* (4). The genomic fragments most commonly chosen for species-specific amplification of the *M. tuberculosis* complex are the insertion sequences IS*6110* (39) and IS*1081* (8) and the MPB70 gene (14, 32). In a recent report, a genomic fragment designated *mtp40* was identified as species specific for *M. tuberculosis* (30) and was amplified by PCR with oligonucleotides PT-1 and PT-2 (18).

This study reports the optimization and evaluation of a multiplex PCR assay designed to distinguish *M. tuberculosis* from other members of the *M. tuberculosis* complex by targeting the *mtp40* and IS*1081* genes in a single reaction. We also report a study by PCR of the host range of IS*6110*, the MPB70 gene, and the 16S rRNA gene in a wide variety of *Mycobacterium* species from different animal and geographic origins. An established immunoperoxidase test for the detection of MPB70 antigen in *M. bovis* strains was also evaluated to determine the value of this test compared with the more recently developed genetic markers.

MATERIALS AND METHODS

Mycobacterial strains. A total of 339 strains of mycobacteria belonging to 31 different species, including reference strains and clinical isolates, from different animal and geographic origins were tested. Two hundred eighty-six strains were from the culture collection of the Australian Reference Laboratory for Bovine Tuberculosis, South Perth, Western Australia, Australia, and 53 were from the Mycobacteria Laboratory, Departamento de Patología Animal I (Sanidad Animal), Universidad Complutense de Madrid, Madrid, Spain. All isolates were

TABLE 2. Nucleotide sequences and expected product sizes of primers pairs used in the PCR study

Primer[a]	Sequence (5′ to 3′)	Product size (bp)
PT-1	CAA CGC GCC GTC GGT GG	396
PT-2	CCC CCC ACG GCA CCG C	
1081-L	TCG CGT GAT CCT TCG AAA CG	238
1081-9	GCC GTT GCG CTG ATT GGA CC	
INS-1	CGT GAG GGC ATC GAG GTG GC	245
INS-2	GCG TAG GCG TCG GTG ACA AA	
TB1-F	GAA CAA TCC GGA GTT GAC AA	372
TB1-R	AGC ACG CTG TCA ATC ATG TA	

[a] PT, *mtp40* primers; 1081, IS*1081* primers; INS, IS*6110* primers; TB1, MPB70 primers.

identified by conventional methods (21). Prior to use in this study the mycobacterial strains were stored at −80°C in Protect Vials (Sigma Pharmaceuticals, Clayton, Victoria, Australia) as recommended by the supplier. The strains were reactivated by removing a single bead coated with organism and adding it to culture media under sterile conditions. The bacterial strains used in this study and their sources are listed in Table 1.

Preparation of crude bacterial suspensions and DNA extracts. Mycobacterial species were cultured on B83 blood agar (9) or Stonebrinks media (38), with the exception of *M. genavense* which was grown on Middlebrook 7H11 (Difco Laboratories, Detroit, Michigan). Cultures were incubated in air at 36°C, except for *Mycobacterium ulcerans* and *Mycobacterium marinum*, which were incubated at 30°C, until growth was evident. A single colony was added to 100 μl of purified water and heated to 94°C for 10 min to expose the DNA for PCR and then stored frozen at −20°C until tested. DNA extractions were performed as described previously (13).

Overall study design. All strains were tested by the multiplex PCR developed for *mtp40* and IS*1081*, by multiplex PCR for 16S rRNA and MPB70, and by PCR for IS*6110*. In some cases ($n = 50$), extracted DNA (approximately 20 ng) was tested in place of the cell suspension. Strains with aberrant results were retested at least three times, and when available, both DNA and cell suspensions were tested in parallel. In addition, all *M. tuberculosis* and *M. africanum* strains that were negative for *mtp40* and more than half of the *M. bovis* strains were also tested for *mtp40* in a simple PCR for *mtp40* sequence as a check on the multiplex results. Two hundred twenty-three of the strains were tested by the immunoperoxidase test.

Oligonucleotide primers. Oligonucleotide primers were synthesized on a Pharmacia Gene Assembler Plus and gel purified on a 20% polyacrylamide gel containing 7 M urea. The oligonucleotides were visualized by UV imaging and desalted on NAP-10 columns (Pharmacia Biotech). The nucleotide sequences and the expected product sizes from the amplification reactions are shown in Table 2.

Amplification conditions for *mtp40*-IS*1081* multiplex PCR. The reaction conditions were designed for a high degree of specificity. A careful titration of each component was required to optimize the PCR to generate two bands of equivalent intensity. Final amplification conditions were as follows: PCR was carried out in a total volume of 25 μl containing 0.5 mM $MgCl_2$, 0.5 U of *Tth* plus polymerase (Biotech International, Bentley, Western Australia, Australia), 60 ng of each IS*1081* primer, 150 ng of each of the PT primers, 1× reaction buffer [67 mM Tris-HCl (pH 8.8 at 25°C), 16.6 mM $(NH_4)_2SO_4$, 0.2 mM deoxynucleoside triphosphate, 1 mM 2-mercaptoethanol, 6 M EDTA, 0.2 mg of gelatin per ml], and 5 μl of the sample. Reaction mixes were overlaid with 50 μl of paraffin oil. After an initial denaturation of 10 min at 94°C the reaction mixes were amplified by 35 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 2 min, and extension at 72°C for 3 min with a Corbett FTS-1 thermal cycler (Biotech International).

Amplification conditions for 16S rRNA-MPB70 multiplex and IS*6110* PCRs. Amplification conditions for the 16S rRNA-MPB70 multiplex PCR were similar to those described previously (46). PCR amplification of the IS*6110* element was carried out in conditions similar to those described for the *mtp40*-IS*1081* multiplex PCR, modified by using 2 mM $MgCl_2$, 1 U of *Tth* plus polymerase, and 100 ng each of the INS-1 and INS-2 primers (23).

Amplification products were analyzed by gel electrophoresis at 100 V for 45 min on 2% agarose gels. Gels were stained in ethidium bromide and photographed on a UV transilluminator.

Immunoperoxidase assay. The immunoperoxidase assay was performed as described previously (10) using a dot blot technique with a crude antigen extract, an autoclaved suspension of organisms with an opacity of 5 (McFarland Opacity Standards; Difco), and 4C3/17, a commercial monoclonal antibody to MPB70 (Agen Biomedicals, Brisbane, Queensland, Australia).

RESULTS

Optimization of PCRs. One of the most important parameters for the specific amplification of the *mtp40* sequence was the concentration of $MgCl_2$. The optimum results were obtained with 0.5 mM of $MgCl_2$; higher concentrations resulted in generation of nonspecific amplification products from *M. bovis*. A minimum number of amplification cycles ($n = 35$) and a high annealing temperature (65°C) were used to minimize any potential cross-reaction with other mycobacterial species. We found that 0.5 U of *Tth* plus DNA polymerase was sufficient to perform the multiplex amplification successfully. During the optimization of the reaction conditions, a multiplex PCR with primers to *mtp40* and to IS*6110* was tried. It was observed, when both pairs of primers were used in a single tube, that the efficiency of the amplification for the element IS*6110* was higher than that encountered for the *mtp40* sequence. This fact resulted in the existence of false-negative results with mistakes in the identification of the isolates. This competition phenomenon was not observed in the multiplex reaction for *mtp40* and IS*1081*. After combined titration of both oligonucleotide pairs, we found that accurate results were obtained with 150 ng of each of the PT primers and 60 ng of each of the 1081 primers.

Table 3 summarizes the results of testing the 339 mycobacterial isolates by PCR for *mtp40*, IS*1081*, 16S rRNA, MPB70, and IS*6110* targets and the immunoperoxidase test for the presence of the MPB70 antigen.

TABLE 3. Results of testing 339 mycobacterial isolates by PCR for different targets and the immunoperoxidase test for the presence of MPB70 antigen

Mycobacterium species	No. of isolates positive/no. tested					
	By PCR for:					By immunoperoxidase test
	mtp40	IS*1081*	16S rRNA	MPB70	IS*6110*	
M. tuberculosis[a]	87/91	91/91	91/91	91/91	87/91	1/36
M. africanum	9/11	11/11	11/11	11/11	11/11	1/11
M. tuberculosis complex from seals	17/17	17/17	17/17	17/17	17/17	0/17
M. bovis	0/161	161/161	161/161	161/161	161/161	110/111
M. bovis BCG	0/17	17/17	17/17	17/17	17/17	17/17
M. microti dassie bacillus	0/7	7/7	7/7	7/7	7/7	0/7
Mycobacterium spp.	0/35	0/35	35/35	0/35	2/35	0/24
Total	115/339	304/339	339/339	304/339	302/339	129/223

[a] Includes three isolates that were susceptible to thiophen-2-carboxylic acid hydrazide and four from Vietnamese patients that were known to lack IS*6110*.

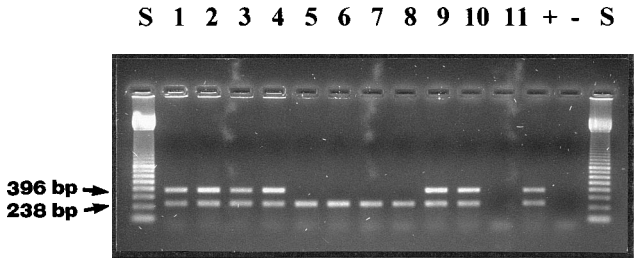

FIG. 1. Multiplex PCR amplification products from various *Mycobacterium* spp. after electrophoresis on a 2% agarose gel and staining with ethidium bromide. Lanes: 1 and 2, *M. tuberculosis*; 3 and 4, *M. africanum*; 5 and 6, *M. bovis*; 7 and 8, *M. microti*; 9 and 10, strains from seals; 11, *M. kansasii*; S, 100-bp ladder (Pharmacia Biotech) used as a DNA size standard; + and −, positive and negative controls, respectively, for the reaction. The bands of 396 and 238 bp indicate the presence of the sequences *mtp40* and IS*1081*, respectively.

PCR testing. The 396-bp fragment for *mtp40* was amplified from the genome of 87 of the 91 (95.6%) isolates of *M. tuberculosis*, from 9 of the 11 (81.8%) strains of *M. africanum*, and from all of the 17 strains from seals tested. The identification of the four isolates of *M. tuberculosis* and the two isolates of *M. africanum* that lacked the *mtp40* sequence was reconfirmed by conventional tests (colony morphology, oxygen preference, niacin production, nitrate reduction, susceptibility to pyrazinamide, and susceptibility to thiophen-2-carboxylic acid hydrazide) (21). The *mtp40* sequence was not detected in any of the strains identified as *M. bovis*, *M. bovis* BCG, *M. microti*, or nontuberculous mycobacteria. The 238-bp fragment for IS*1081* was amplified from the genomes of all 304 *M. tuberculosis* complex members tested but not from any of the other species of mycobacteria. Figure 1 shows the amplification products generated from various species of mycobacteria by multiplex PCR for the elements *mtp40* and IS*1081.*

In the 16S rRNA-MPB70 PCR, the 1,030-bp genus-specific product was generated from all of the 339 mycobacterial isolates, and all of the 304 strains of the *M. tuberculosis* complex produced the 372-bp amplification product for MPB70.

The presence of the IS*6110* sequence was investigated by PCR in the 339 mycobacterial isolates and was found in 300 of the 304 *M. tuberculosis* complex strains tested. The strains that lacked IS*6110* were all *M. tuberculosis* isolates from Vietnamese patients. This sequence was also detected in the strains of *M. ulcerans* and *Mycobacterium gilvum* that were included in our study.

Immunoperoxidase test. All but 1 of the 111 strains identified as *M. bovis* and all of the 17 *M. bovis* BCG strains tested were positive when tested by the immunoperoxidase test for MPB70 antigen (Table 3). All of the *M. microti* strains and all of the strains from seals were negative by the immunoperoxidase test. One isolate identified as *M. tuberculosis* and one identified as *M. africanum* were positive by the immunoperoxidase test.

DISCUSSION

The aim of this study was to develop a single-tube reaction that would be able to detect the presence of the IS*1081* element (specific for *M. tuberculosis* complex organisms) and then to identify the species *M. tuberculosis* by means of the presence of the *mtp40* sequence. Although the genomic fragment *mtp40* was originally described as species specific for *M. tuberculosis* (18, 30) and results of a recent study using a limited number of strains were consistent with these findings (35), we found that the *mtp40* fragment is also present in *M. africanum* and in the strains from seals. The presence of this element in 9 of the 11 *M. africanum* strains tested supports the existing controversy in the classification of these isolates. *M. africanum* is a highly heterogeneous group of mycobacteria reported to be phenotypically intermediate between *M. bovis* and *M. tuberculosis*. In a numerical taxonomy study of 60 *M. africanum* isolates from five different African countries, these strains clustered with *M. tuberculosis* or *M. bovis* according to their geographic origin (17).

The strains from seals tested were originally identified as *M. bovis* by means of biochemical and drug susceptibility tests (12). However, these strains lacked detectable amounts of MPB70 antigen, considered to be virtually specific for *M. bovis* isolates (42, 47), when tested with the immunoperoxidase assay. After genomic characterization by restriction endonuclease analysis and restriction fragment length polymorphism, the strains from seals formed a unique cluster clearly different from the bovine *M. bovis* strains, and they seemed to be more closely related to *M. tuberculosis* than to *M. bovis* (12). The presence of the *mtp40* fragment in the strains from seals supports the genetic relationship of these isolates with the species *M. tuberculosis.*

Some researchers have recently reported the existence of *M. bovis* strains from human patients with the *mtp40* sequence (40a); however, in the present study none of the 42 *M. bovis* strains of human origin tested showed the presence of this element.

The identification of the four *M. tuberculosis* and the two *M. africanum* strains that lacked the *mtp40* sequence was confirmed by standard biochemical tests. We did not find any correlation between the variety of strain and the presence of the element. Two of the *M. tuberculosis* strains were susceptible (Asian variety) and two were resistant (classical variety) to thiophen-2-carboxylic acid hydrazide. One of the *M. africanum* strains was confirmed as type I, and the other was confirmed as type II.

A previous investigation of the host range of IS*1081* revealed that, apart from the species of the *M. tuberculosis* complex, it was also present in strain TMC 1470 (Trudeau Collection, National Institutes of Health, Bethesda, Md.) of *Mycobacterium xenopi* (7). Our study included the analysis of four clinical isolates and two type strains of *M. xenopi*, and all of them lacked the IS*1081* element.

Recently, some authors (41, 49) have reported the existence of *M. tuberculosis* strains that lack the IS*6110* sequence; such strains have originated from Southeast Asia and India. In this study, four *M. tuberculosis* isolates from Vietnamese patients lacked the IS*6110* element. However, to our knowledge, no members of the *M. tuberculosis* complex have been reported to lack IS*1081*. This suggests that the IS*1081* element is a good option for inclusion in the multiplex PCR to identify *M. tuberculosis* complex bacteria, especially in Southeast Asia. IS*6110* had not previously been reported to occur in mycobacteria other than *M. tuberculosis* complex until a recent report (27) of homology between IS*6110* and six other *Mycobacterium* spp. by a nested PCR technique. In our study a PCR product consistent with IS*6110* was detected in one strain of *M. ulcerans* and in one strain of *M. gilvum*. These strains were retested four times, and negative controls were included with each batch of PCR tubes in order to detect possible cross-contamination. For retesting the strains we selected different subculture tubes as well as the original strain held in our culture collection. These findings emphasize the importance of more extensive investigation of the host range of this sequence in order to prevent both false-positive and false-negative results in the identification by PCR of *M. tuberculosis* complex organisms.

The immunoperoxidase test was found to be a simple, reliable, and specific method for identification of *M. bovis* strains. The one strain that was originally identified as *M. bovis* but was negative by the immunoperoxidase test was isolated from a human patient who had been born in Singapore. When reassessed by biochemical tests, the strain was intermediate between *M. bovis* and *M. tuberculosis* but more closely resembled *M. tuberculosis*. This particular strain also lacked *mtp40*, which in this study was more consistent with *M. bovis.*

By the *mtp40*-IS*1081* multiplex PCR, most *M. africanum* strains have grouped with *M. tuberculosis*, giving a positive test for the human pathogens, whereas the traditional animal pathogens *M. bovis* and *M. microti* gave a negative result. The exception to this finding is the strains from seals, which grouped with the human pathogens. At present, there is no single rapid method for species identification within the *M. tuberculosis* complex. We suggest that the PCR reported here could be used with other tests, including the immunoperoxidase test and biochemical tests, for identification of members of the *M. tuberculosis* complex.

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